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Short communication

Characterization of *Toxoplasma gondii* isolates from free range chickens from Paraná, Brazil

J.P. Dubey [a,*], I.T. Navarro [b], D.H. Graham [c], E. Dahl [c], R.L. Freire [b], L.B. Prudencio [b], C. Sreekumar [a], M.C. Vianna [a], T. Lehmann [c]

[a] *Animal Parasitic Diseases Laboratory, United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, BARC-East, Building 1001, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, USA*
[b] *Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Campus Universitário-Caixa Postal 6001 CEP 86051-990-Londrina, Paraná, Brazil*
[c] *Division of Parasitic Diseases of Centers for Disease Control and Prevention, 4770 Buford Highway, MS:F22, Chamblee, GA 30341, USA*

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Abstract

The prevalence of *Toxoplasma gondii* in free range chickens is a good indicator of the prevalence of *T. gondii* oocysts in the environment because chickens feed from the ground. In the present study, prevalence of *T. gondii* in 40 free range chickens (*Gallus domesticus*) from a rural area surrounding Paraná, Brazil was assessed. Blood, heart, and brain from each chicken were examined for *T. gondii* infection. Antibodies to *T. gondii*, assayed with the modified agglutination test (MAT $\geq$ 1:5) were found in 16 chickens. Hearts and brains of seropositive (MAT $\geq$ 1:5) chickens were bioassayed in mice. Additionally, hearts and brains of seronegative (MAT $<$ 1:5) chickens were bioassayed in two *T. gondii*-free cats (12 chickens per cat). *T. gondii* was isolated from 13 of 16 (81%) seropositive chickens. Of the two cats fed tissues pooled form seronegative chickens, one shed *T. gondii* oocysts. Nine of the 13 *T. gondii* isolates killed 100% of infected mice. The *T. gondii* isolate from the cat was also virulent for mice. Genotyping of 13 chicken isolates of *T. gondii* using the SAG2 locus indicated that seven isolates were type I and six were type III; three of these type III isolates killed all infected mice suggesting that all strains virulent for mice are not type I. The isolate from the feces of the cat fed chicken tissues was type I.
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Keywords: *Toxoplasma gondii*; Toxoplasmosis; Isolation; Genotyping; Chickens; *Gallus domesticus*; Paraná, Brazil

* Corresponding author. Tel.: +1-301-504-8128/8984; fax: +1-301-504-9222/6273.
E-mail address: jdubey@anri.barc.usda.gov (J.P. Dubey).

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1. Introduction

Toxoplasma gondii infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected postnatally mainly by ingesting tissue cysts from undercooked meat or from the food or drink contaminated with oocysts shed in cat feces. However, only a small percentage of exposed adult humans or animals develop clinical signs. It is unknown whether the severity of *T. gondii* infections is due to the parasite strain, infectious dose, host immunity, or other factors. Overall, there is low genetic diversity among *T. gondii* isolates so far examined. *T. gondii* strains have been classified into three genetic types (I, II, III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995; Howe et al., 1997).

It has been suggested that type I isolates or recombinants of types I and III are more likely to result in clinical toxoplasmosis (Howe et al., 1997; Grigg et al., 2001; Fuentes et al., 2001; Aspinall et al., 2003), but genetic characterization has been limited essentially to patients ill with toxoplasmosis. Contrary to those of humans, most isolates of *T. gondii* obtained from animals and genetically typed were type II or type III, irrespective of the clinical status of the animal (Howe and Sibley, 1995; Mondragon et al., 1998; Owen and Trees, 1999; Jungersen et al., 2002). As chickens become infected mostly by feeding from the soil contaminated with oocysts, prevalence of *T. gondii* in chickens is a good indicator of prevalence of *T. gondii* in their environment (Ruiz and Frenkel, 1980). We have chosen free range chicken to examine genetic diversity of *T. gondii* from many countries (Dubey et al., 2002b, 2003a,b,c,d).

Until recently, most of the *T. gondii* isolates genetically typed were from Europe and the US (Dubey et al., 2002b). Recently, 51 of 73 isolates of *T. gondii* obtained from asymptomatic free range chickens from rural areas surrounding São Paulo and Rio de Janeiro, Brazil were classified type I (Dubey et al., 2002b, 2003b). Contrary to these report, *T. gondii* isolates of chickens from Egypt (Dubey et al., 2003a) and the US (Dubey et al., 2003c; Lehmann et al., 2003) were type III. The purpose of this study was to isolate *T. gondii* from chickens from another region of Brazil and to genetically characterize them.

2. Materials and methods

2.1. Naturally infected chickens

The chickens were free range, about 1 year old, and were obtained from rural areas of Santa Isabel do Ivaí (latitude 23°00′00″, longitude 53°11′50″), Paraná State, Brazil. They were purchased from 10 different rural houses approximately 100–200 m apart. Chickens were bled and killed on 13 October 2002. Heart, head, and serum from each bird were transported by air to the USDA's laboratory in Beltsville, MD for *T. gondii* examination. Four days elapsed between killing and examination for *T. gondii* and during this time samples were kept cold, but not frozen.

2.2. Serologic examination

Sera from chickens were diluted two-fold starting at 1:5 dilution and assayed for *T. gondii* antibodies with the modified agglutination test (MAT) as described (Dubey and Desmonts, 1987).

2.3. Bioassay of chicken tissues for T. gondii infection

The procedures were identical to those described by Dubey et al. (2002b, 2003b). Brains and hearts of seropositive (MAT $\geq$ 1:5) chickens were bioassayed individually in mice after digestion in pepsin. For this, brain and heart of each chicken were pooled, homogenized in five volumes (w/v) of aqueous 0.85% NaCl (saline), mixed with five volumes of acidic pepsin and the mixture incubated in a shaker water bath for 1 h at 37 °C. The digest was centrifuged, neutralized, mixed with antibiotics, and the homogenate was inoculated subcutaneously (s.c.) into five mice. The mice used were Swiss Webster albino females obtained from Taconic Farms (Germantown, New York); spontaneous *T. gondii* infection has never been documented in the thousands of these mice used for bioassays in our laboratory. Tissue impression smears of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 45 post-inoculation (p.i.), and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 50 days p.i. and their brains were examined microscopically for tissue cysts, and a portion of the brain was frozen for DNA extraction. Mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were demonstrable in their tissues.

Hearts and brains from 24 seronegative (MAT $<$ 1:5) chickens were pooled in batches of 12 chickens and fed to two *T. gondii*-free cats. Feces of cats were examined for *T. gondii* oocyst shedding as previously described (Dubey, 1995). Oocysts were suspended in 2% H_2SO_4, allowed to sporulate at room temperature, and bioassayed in mice as described previously (Dubey and Beattie, 1988).

2.4. Genetic characterization

T. gondii DNA was extracted form infected mouse tissues as described by Lehmann et al. (2000). The PCR-RFLP genotypes of SAG2 locus were used to determine the genetic type (Howe et al., 1997). Briefly, the DNA fragments at the 3′ and 5′ ends of the SAG2 gene were amplified using a nested PCR. They were digested with restriction enzymes *Sau* 3A1 and *Hha* 1, respectively, and the digestion products were size fractionated on a 1.5% agarose gel. The presence of a restriction site at the 3′ fragment indicated type III and that on the 5′ fragment indicated type II. Lack of digestion on both fragments indicated type I.

3. Results

Antibodies to *T. gondii* (MAT $\geq$ 1:5) were found in 16 of 40 (40%) chickens. The MAT titers of chickens were 1:5 in three, 1:10 in one, 1:20 in three, 1:40 in one, 1:80 in two, 1:160 in two, 1:320 in one, 1:640 in one, and 1:1280 or more in two.

Table 1
Isolation of *T. gondii* from chickens from Santa Isabel do Ivaí, Paraná, Brazil

Chickens		Bioassay in mice				
Number	Titer	Group number	Number of mice *T. gondii* positive[a]	Number dead	Day of death	Genotype
2	1:10	1	5	0	0	III
7	1:5	3	5	1	25	III
8	1:640	4	5	5	15–21	III
12	≥1:1280	5	2	0	0	I
13	1:160	6	5	5	15–26	I
20	≥1:1280	9	4	4	15–19	I
23	1:20	10	4	4	13–22	III
25	1:320	11	5	5	13–20	III
34	1:80	12	5	5	15–30	I
36	1:160	13	3[b]	3[b]	15–21	I
38	1:80	14	5	5	13	I
39	1:20	15	5	5	11 or 13	I
40	1:40	16	5	0	0	III

[a] Of five mice inoculated.
[b] Two mice were badly autolyzed and not examined.

T. gondii was isolated from tissues of 13 of 16 seropositive chickens (Table 1). Nine isolates killed all infected mice between 10 and 26 days (Table 1).

One cat fed tissues of 12 seronegative chickens shed 9 million *T. gondii* oocysts. The mice fed sporulated oocysts from this cat died of acute toxoplasmosis 4 days later; the mice that were subpassaged s.c. with tachyzoites recovered from the mesenteric lymph nodes died 5 days later. Two cats fed tissues of mice acutely infected with *T. gondii* isolates from chicken nos. 38 and 39 shed 75 and 270 million oocysts; these oocysts were lethal to mice, even those fed last dilution (representing a single oocyst) from titrated inocula. Of the 13 isolates of *T. gondii* from chickens obtained by mouse bioassay and genotyped individually, seven were type I and six were type III. The isolate obtained by bioassay in the cat was type I. Mixed genotypes were not identified.

4. Discussion

In the present study, mice inoculated with tissues of chickens naturally-infected with type I isolates survived at least 10 days. Because tissue cysts are formed in mice as early as 8 days p.i., irrespective of the route of inoculation or the stage of *T. gondii* inoculated (Dubey et al., 1998), ingestion by cats of even acutely infected mice can induce oocyst formation. Thus, it is confirmed that type I isolates can circulate in nature at least by an avian-cat cycle. Until recently, it was presumed that type I isolates of *T. gondii* do not produce many oocysts (see Dubey et al., 2002b, 2003b). Results of the present study and that reported earlier (Dubey et al., 2002b) indicate that type I isolates can induce shedding of large numbers of oocysts in cats.

Results of the present study confirm our earlier observations that asymptomatic chickens from Brazil can harbor *T. gondii* type I strains virulent for mice (Dubey et al., 2002b, 2003b). In the present study, chickens were from rural Paraná, whereas in the previous study chickens were from rural São Paulo and Rio de Janeiro; these localities are approximately 500 km apart. Thus, these isolates from São Paulo, Rio de Janeiro and Paraná are likely to be genetically independent. We cannot be sure if all isolates of *T. gondii* from Paraná are independent as the houses were close to each other and the exact location of each chicken was not recorded. Although, the study is leased on only one locus (SAG2), there was no evidence for mixed genotypes in chickens.

All *T. gondii* isolates of type I so far studied were found to be virulent to outbred mice (Dubey et al., 2002b). However, mouse virulence has rarely been documented in isolates on their primary isolation in mice. Therefore, we documented mouse mortality data of isolates of *T. gondii* from chickens on their primary isolation. In the present study, 9 of the 13 isolates obtained by bioassay in mice killed all infected mice. Of interest is that three of the type III isolates also killed 100% of infected mice confirming our earlier observations that even type III isolates from Brazil can be lethal to mice (Dubey et al., 2002b, 2003b). In addition, the type I isolate from chicken 12 did not kill mice. With respect to virulence in mice, these *T. gondii* isolates from chickens from Brazil are phenotypically different from isolates of *T. gondii* from pigs and chickens from the US (Dubey et al., 1995, 2002a, 2003b; Lehmann et al., 2003); none of the isolates from chickens or pigs from US were virulent for mice. Studies are now in progress to genetically compare the isolates of *T. gondii* from free range chickens from other countries.

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